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*In: NUTRIHORT: Nutrient management, innovative techniques and nutrient legislation in intensive horticulture for an improved water quality. 2013, Ghent - Belgium.*

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**Conference Website:**

<http://www.ilvo.vlaanderen.be/nutrihort>

**Date deposited:**

04/08/2017

## (42) Method using gas chromatography mass spectrometry (GC-MS) for analysis of nitrate and nitrite in vegetables

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**Abstract:** Accurate analytical methods are very important to detect the contents of chemical substances in vegetables and fruit. There is particular interest in nitrate and nitrite, due to their possible benefits and harm on human health. The aim of this research is to modify the method of measurement of nitrate and nitrite by GC-MS for use in vegetables. Many procedures are available to measure nitrate (NO<sub>3</sub>) and nitrite (NO<sub>2</sub>). Some examples are: capillary electrophoresis, spectrophotometry using the Griess reaction, various HPLC methods and different types of isotope dilution GC-MS: with electron ionisation by nitration of mesitylene, and with chemical ionisation by derivatisation with pentafluorobenzyl bromide. However, some of these methods are mostly used in research on human health, to measure nitrate and nitrite in human biological fluids such as plasma or urine. Methods developed for analysis of vegetable samples often give variable results, for example due to interference from coloured compounds in the plant material. Therefore, we tried to modify the GC-MS method using pentafluorobenzyl bromide and chemical ionisation to optimise it for analysis of nitrate and nitrite in vegetable samples. Experiments were carried out to maximise peak area, in particular for nitrate. Factors that were tested were gas pressure, derivatisation reaction time and temperature, duration of nitrogen flushing, time of vortex after adding toluene during sample preparation, and amount of water added to separate the phases. The modified method was used to analyse contents of nitrate and nitrite in samples from a range of different vegetables.

**Keywords:** Nitrate; Nitrite; GC-MS modified; Vegetables

### Introduction

Vegetables are important constituents of a healthy diet. Adequate daily eating of vegetables can help reduce the major risk of diseases, including cardiovascular diseases, cancers, obesity and diabetes (World Health Organization, 2003). In addition, vegetables are also naturally rich in nitrate (Lundberg et al., 2004). Intervention studies have shown that short-term intake of inorganic nitrate decreases blood pressure in healthy volunteers (Siervo et al., 2013). The main risk of nitrate in vegetables is from its conversion to nitrite, creating methaemoglobin by reacting with haemoglobin after ingestion, where the foetal haemoglobin in infants is particularly susceptible (Greer and Shannon, 2005).

Therefore, the determination of the nitrate and nitrite levels in vegetables is important in order to evaluate their safety for consumers (Correia et al., 2010). Analytical methods have an important role in nitrate and nitrite analysis of vegetable samples and there are several procedures available. For example, capillary electrophoresis can be applied to measure high amounts of nitrate in vegetables. However, the samples must be diluted because of interfering oxalate in the same vegetables such as in spinach and tomato (Jimidar et al., 1995). The most commonly used method is the colorimetric method based on the Griess reaction. This method is very popular and of low cost but it has a number of drawbacks. It is only accurate within a narrow concentration range and is therefore difficult to use for measuring the very variable nitrate content in vegetables. Also, the colour reaction is susceptible to interference from the dark colour in samples such as beetroot, red chard and cabbage. Additionally, a standard curve is needed in every test session because the changes in any conditions such as temperature affect the results. In addition, there is interference between nitrate and nitrite. Several other methods were developed for analysis of nitrate and nitrite in human samples, but they require more modification to become suitable for analysis of vegetables. Among them a GC/MS method using pentafluorobenzyl bromide (PFB-Br) and <sup>15</sup>N-labelled nitrite and nitrate as internal standards is very accurate and useful to investigate nitrite and nitrate metabolism and reactions in the human body (Tsikas, 2000). However, this method was developed for human fluids. This method measures the nitrate and nitrite contents simultaneously in the same sample based on isotope dilution. The isotope dilution means that the measured concentration values depend on the relative sizes of peaks with different molecular mass, not on the absolute size of the peaks. This makes the method exceptionally robust in relation to any external factor affecting peak size. Measurements are accurate across a range of

more than 3 orders of magnitude, as long as the peaks are large enough for accurate measurement of their areas, and the volumes of sample and spiking solution are measured accurately.

The main aim of this study is to modify the PFB-Br GC/MS method to develop an improved method for analysis of nitrate and nitrite in vegetable samples.

## Materials and Methods

### Sample preparation:

Several vegetable samples (radish, tomato, red cabbage, red chard, mizuna, lettuce and beetroot) were used in this study; some of them have high nitrate content and others have low nitrate content. All samples were prepared by homogenising approximately 14 to 16g of vegetables mixed with 15-25ml of de-ionized water using an Ultra TurraxT-25 for at least 1 minute and allowing the separation of residue and sample solution by centrifugation.

A high amount of plant materials per sample was used to obtain a high concentration of nitrate and nitrite in the extracts and to reduce random variation caused by variable concentrations in different parts of the sample (Anderson and Case, 1999). Direct homogenisation of fresh frozen samples prevented potential problems related to insufficient hydration of dry material or sequestration of nitrate in intact plant cells.

### Reagent and Chemicals:

Sodium [ $^{15}\text{N}$ ]Nitrate (15N, 98%+) and Sodium [ $^{15}\text{N}$ ]Nitrite (15N, 98%+) from Cambridge Isotope Laboratories, Inc. (USA). 2,3,4,5,6-Pentafluorobenzyl bromide 99% from Sigma-Aldrich (Germany). Nitrogen compressed gas (oxygen free) and methane (low ethylene) from BOC gases (UK). Toluene (reagent grade) and acetone (general purpose grade) from Fisher Scientific (UK).

For each of the following steps, two or more variants were tested to determine which version would give the best results with vegetables: gas pressure, derivatisation reaction time and temperature, duration of nitrogen flushing, time of vortex after adding toluene during sample preparation, and amount of water added to separate the phases. In each case by analysing duplicate subsamples of 5-10 vegetable extracts with each method in one session. The volumes of sample and all reagents were doubled compared with the original method, since sample volume is not a limiting factor for vegetable samples. Other variables such as column type, ionisation temperature etc. were as described by Tsikas (2000).

The aim of each comparison was to maximise the peak area of nitrate, because this determines the sensitivity of the method (how low concentrations can be measured) and the precision (random variation).

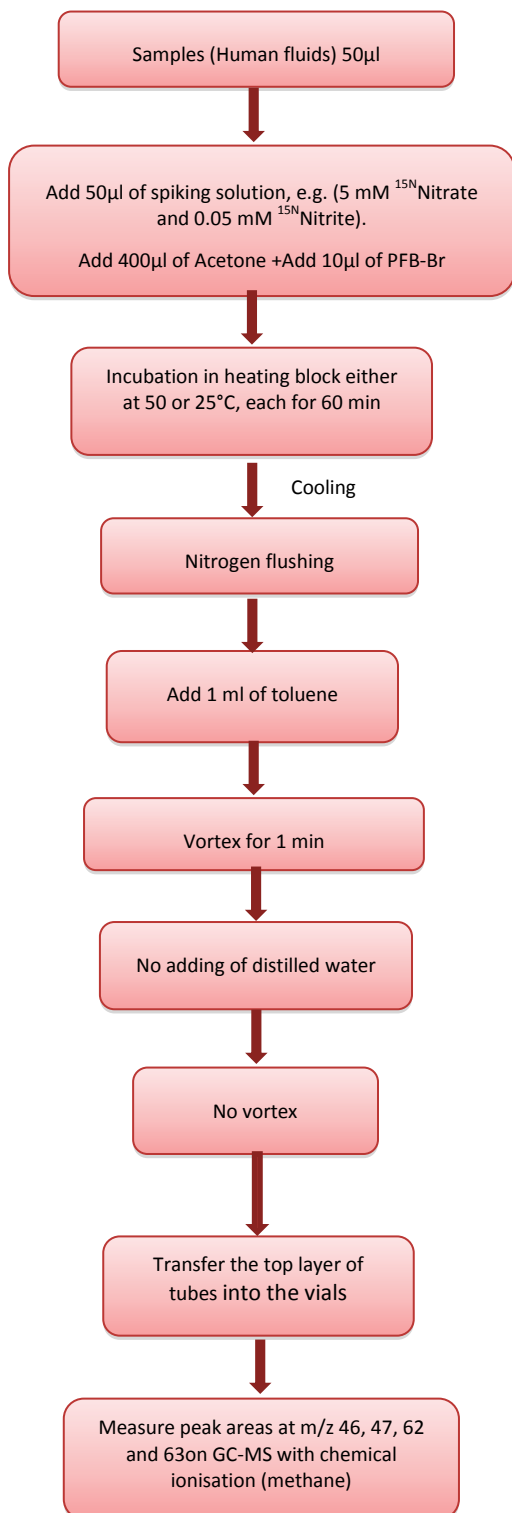
The nitrate content was calculated by the following equation:

$$\text{Nitrate content (mM)} = 5 \cdot [^{14}\text{N}] \text{ nitrate peak area} / [^{15}\text{N}] \text{ nitrate peak area}$$

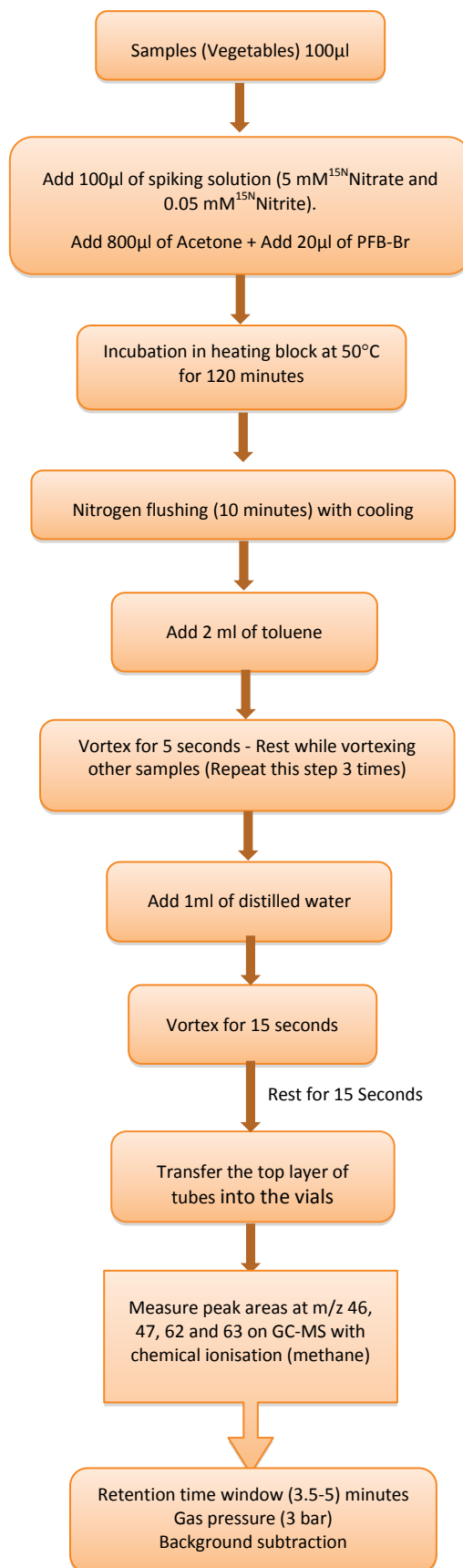
Statistical analysis was performed using the Minitab 16 software. The data were analysed using analysis of variance (ANOVA General Linear Model) and logarithmic transformation of the data. The results were presented as the back-transformed mean + SE (standard error of the mean), and significance at  $p < 0.05$ .

# Sample preparation procedure for GC-MS:

## Tsikis' method



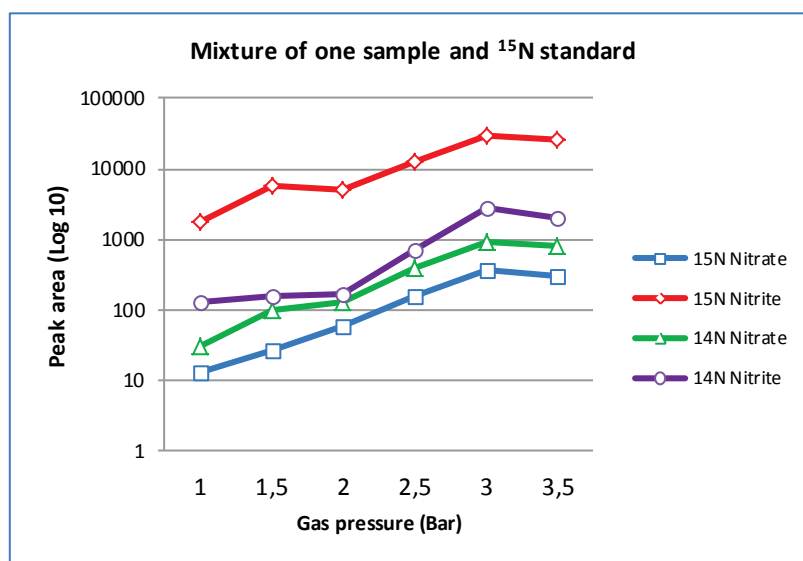
## Modified method



## Results and Discussion

### Gas pressure:

The pressure of the methane ionisation gas is important for the size of the peaks in the GC/MS chromatogram. Due to this, we adjusted the gas pressure through testing of different gas pressures from low (1 bar) to high (3.5 bar). The best result was obtained with 3 bar for both nitrate and nitrite (Figure 1).



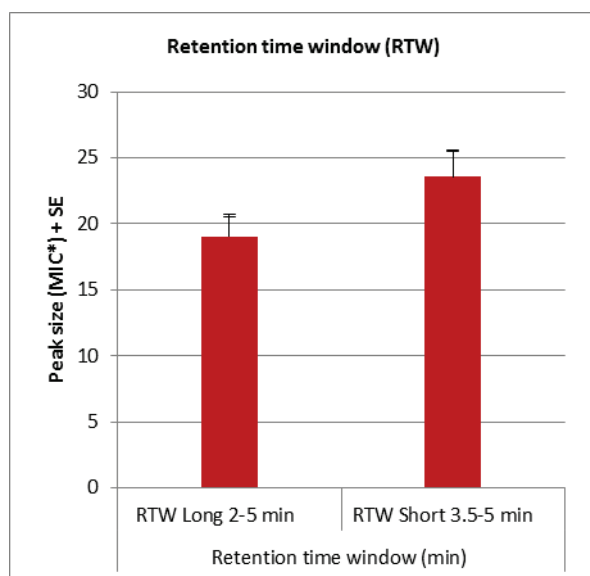
**Figure 1.** The effect of gas pressure on the sizes of nitrate and nitrite peaks in the GC/MS chromatograms

### Retention time window:

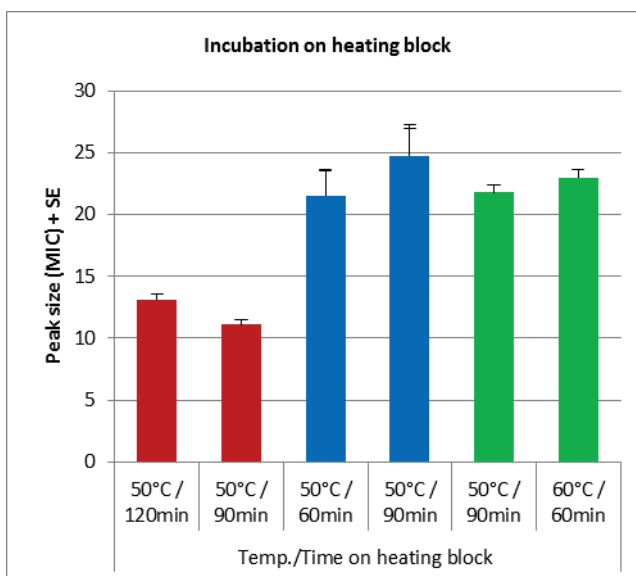
The retention time window interval where chemical ionisation takes place during the analysis was modified through comparing different retention time windows before the first peak (which comes at 3.6-3.8 minutes). There was a significant difference between the long retention time window (RT Long 2-5 minutes) and short retention time window (RT Short 3.5-5 minutes), as shown in Figure 2.

### Incubation:

Reaction time has an effect on the making of PFB-NO<sub>3</sub> and PFB-NO<sub>2</sub> from PFB-Br during incubation on the heating block. Optimal times and temperatures of incubation on heating block were determined in three different experiments through comparing different time periods and temperatures; 50°C/120min versus 50°C/90min; 50°C/60min versus 50°C/90min; and 50°C/90min versus 60°C/60min. The results were that there was no significant difference between 50°C/90min and 60°C/60min. On the other hand, there was a highly significant difference in results for 50°C/120min and 50°C/90min and we found that the (50°C / 120min) treatment is the best condition for formation of PFB-NO<sub>2</sub> and in particular PFB-NO<sub>3</sub>. However, there was also a highly significant difference in experiment of 50°C/60min and 50°C/90min but here the peak areas of nitrite were very small so these conditions were not useful for our experiment (Figure 3).



**Figure 2.** The difference between long and short retention time windows of GC-MS. \*MIC: Multi ion current.



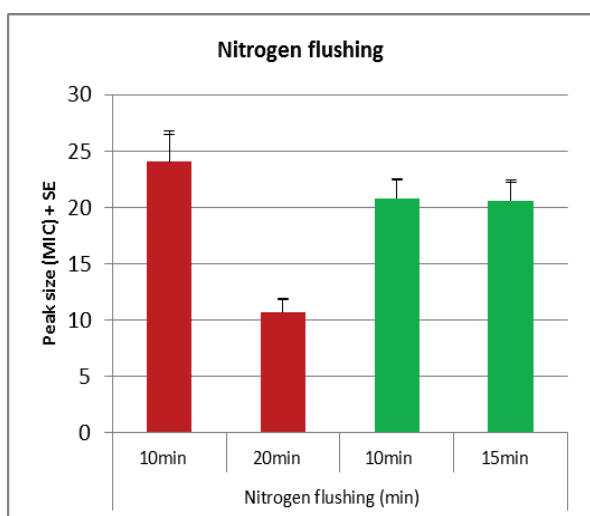
**Figure 3.** The effect of different time and temperature during the incubation step of sample preparation for GC-MS

#### Nitrogen flushing:

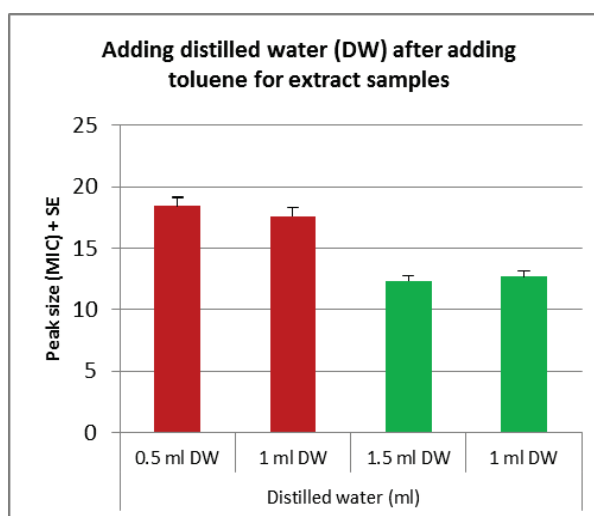
Dry nitrogen flush was used to remove the acetone after the derivatisation reaction was complete. However, too much or too little flushing gave samples where it was difficult to separate the phases afterwards. For this reason, we tried different periods of nitrogen flushing. Figure 4 shows that there was a highly significant difference between 10 and 20 minutes of nitrogen flushing; the best results were obtained with 10 minutes because if the mixture resides for longer time it will get dry and it may make other interfering organic compounds. No change was observed when comparing between 10 and 15 minutes of nitrogen flushing.

#### Adding distilled water (DW):

Adding distilled water (DW) was introduced to make it easier to separate the organic phase from the extracted samples before transferring the top layer to vials, without interfering with the reaction with acetone. The observations of adding different amounts of DW for first experiment (0.5 and 1 ml of DW) showed no significant differences. The results were the same for the second experiment (1.5 and 1 ml of DW). As a result, 1 ml of DW was chosen since it gave us a sufficient amount of supernatant phase after toluene evaporation (Figure 5).



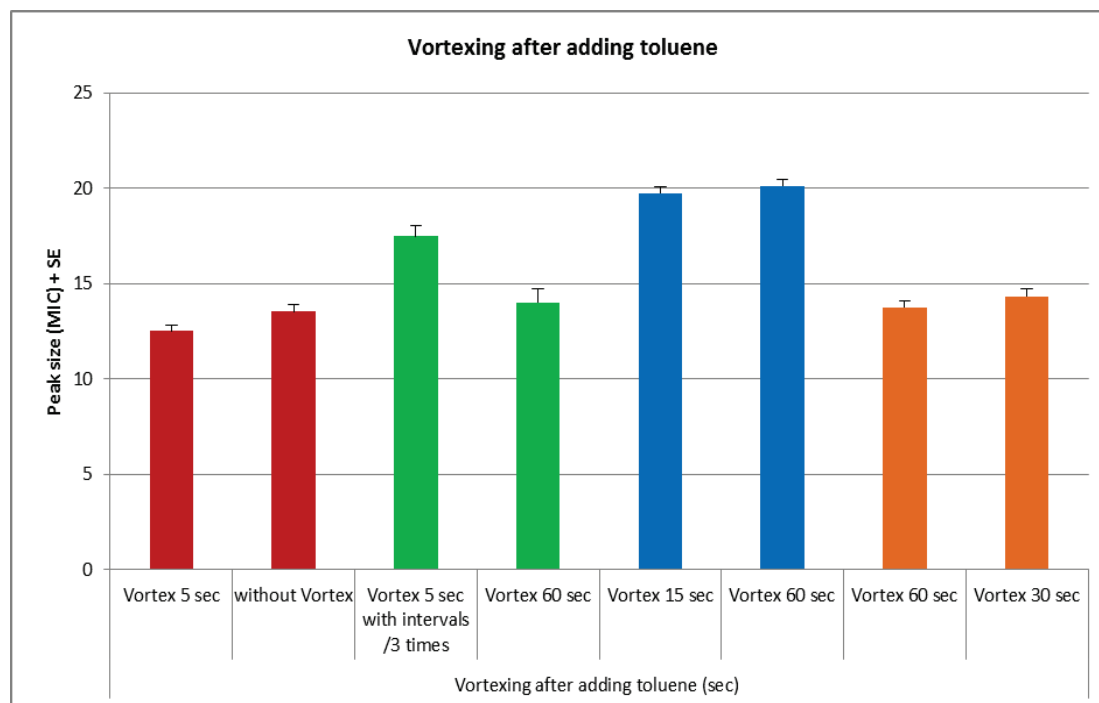
**Figure 4.** The effect of different time of nitrogen flushing



**Figure 5.** The effect of amount of distilled water added

#### Vortex after adding toluene:

Vortex mixing for samples is important to complete the chemical reaction among chemical material and different phases during sample preparation, e.g. after adding toluene or other materials. There was a highly significant difference between vortexing for 5 seconds and without vortex; the peaks of nitrite were particularly small without vortexing. On the other hand, there was no significant difference for vortexing for 60 sec and 15 sec and vortexing for 60 sec and 30 sec. The difference in effect of interval vortexing for 5 seconds and resting between vortex periods repeating 3 times was highly significant compared with vortexing for 60 seconds. This method of mixing was better than other methods tested (Figure 6).



**Figure 6.** Mean of values for vortexing samples after adding toluene

#### Examples of measurement of contents of nitrate and nitrite:

In Table (1) and (2) shows a few examples of measurements by the GC/MS modified method for different types of vegetables. The nitrate content of samples was calculated from the ratios of the peak areas with known concentrations of [ $^{15}\text{N}$ ]nitrate. The mass spectrometric detection was obtained in selected-ion monitoring (SIM). Since the commercially available [ $^{15}\text{N}$ ]nitrate contains a small amount of [ $^{14}\text{N}$ ]nitrate, a blank sample can be used to determine this background content and subtract it before calculating the concentration in the sample. This is important particularly in vegetables that have a low content of nitrate such as tomato. Additionally, nitrite content can be measured in the same way as nitrate in vegetables.

**Table 1.** Examples of recorded data including peak areas (SIM) of nitrate and nitrite in the GC/MS chromatograms in different types of vegetables.

Vegetables	Weight of sample (g)	Total volume (ml)	$^{15}\text{N}$ Nitrate peak area	$^{15}\text{N}$ Nitrite peak area	$^{14}\text{N}$ Nitrate peak area	$^{14}\text{N}$ Nitrite peak area
Blank			5420	12123	310	1479
Tomato	15.67	50	2893	8233	183	3414
Lettuce	20.20	40	11124	6685	89386	2708
Radish	15.25	50	7465	8043	16514	6410
Rocket	15.57	50	4840	9339	28963	2527
Red Cabbage	15.08	50	1715	2268	405	159
Mizuna	15.42	50	1721	3285	8337	514

**Table 2.** Examples of calculations of the amount of nitrate contents in samples and vegetables.

Vegetables	In Samples			In vegetables	
	Nitrate content (mM)	Nitrate content adjusted (mM)	Nitrate content (mg/kg)	Nitrate content (mM)	Nitrate content (mg/kg)
Blank	0.29				
Tomato	0.32	0.03	1.88	0.1	6.0
Lettuce	40.18	39.89	2473	79	4898
Radish	11.06	10.77	668	35.3	2190
Rocket	29.92	29.63	1837	95.2	5900
Red Cabbage	1.18	0.89	55.5	3.0	184
Mizuna	24.22	23.94	1484	77.6	4811

Calculated using data from Table 1

## Conclusion

The modified GC-MS method is a very accurate and relatively rapid method for the determination of high and low contents of nitrate and nitrite in various vegetables. Due to its large dynamic range, there is no need for extensive dilution of samples, irrespective of initial nitrate content. There was no interference with other compounds, which can occur when using the Griess method. However, the method has a limited capacity (72 samples per 24 hours) and the equipment is very expensive.

## Acknowledgements

This work was funded by the Higher Committee of Education Development in Iraq (HCED).

The research assistance of Mr. Christopher Johnson is gratefully acknowledged.

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